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Short communication

# Detection of koi herpesvirus DNA in river water in Japan

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Koi herpesvirus (KHV) has a diameter of 170–230 nm and possesses a double-stranded DNA genome (Pokorova, Vesely, Piackova, Reschova & Hulova 2005). KHV was first discovered in the USA in 1998, followed by outbreaks in koi, *Cyprinus carpio koi* and common carp, *C. carpio carpio*, in Israel and the USA (Hedrick, Gilad, Yun & Spangenberg 2000). The virus subsequently spread to numerous countries worldwide (Haenen, Way, Bergmann & Ariel 2004).

In Japan, the first large outbreak of KHV occurred in October 2003 in Kasumigaura Lake, where more than half of Japan's farmed carp are produced (Sano, Ito, Kurita, Yanai, Watanabe, Miwa & Iida 2004). Subsequently, many outbreaks were reported in almost all of the prefectures in Japan, probably due to transport of infected carp from Kasumigaura Lake to other lakes, ponds or rivers (Kimiya 2004).

Koi herpesvirus is suspected to be transmitted via water as the virus is excreted with faeces of the infected carp (Dishon, Perelberg, Bishara-Shieban, Ilouze, Davidovich, Werker & Kotler 2005). The virus can grow in carp at water temperatures from 15 to 25 °C (Gilad, Yun, Adkison, Way, Willits, Bercovier & Hedrick 2003), thus KHV infection is suppressed during winter months. However, outbreaks of KHV can reoccur the following spring, partly because the virus remains infective in water for a long period (Perelberg, Smirnov, Hutoran, Diamant, Bejerano & Kotler 2003). Before resuming koi farming after an outbreak, it is important to confirm the absence of KHV in the water source.

In our previous studies, we succeeded in detecting various kinds of human enteric viruses, such as noroviruses, adenoviruses or enteroviruses, in aquatic environments (Katayama, Shimasaki & Ohgaki 2002; Haramoto, Katayama, Oguma & Ohgaki 2005). A key step in the procedure is concentration of virus particles ($< 1$ mL concentrated virus sample from 100 to 1000 mL of water sample). Viruses were then detected by polymerase chain reaction (PCR) amplification of the concentrated sample. This method may be more broadly applicable to virus detection in aquatic environments and was used in this study to detect KHV.

The diagnosis of KHV infection is usually based on virus isolation using KF-1 cells, followed by amplification of viral DNA using the PCR technique (Pokorova *et al.* 2005). However, due to the limited susceptibility of KF-1 cells, it is sometimes difficult to isolate KHV even from tissues with high titres of KHV, such as the gill, kidney and spleen of carp (Pokorova *et al.* 2005). Recently, a real-time quantitative TaqMan PCR system was developed for rapid, sensitive and specific detection of KHV (Gilad, Yun, Zagmutt-Vergara, Leutenegger, Bercovier & Hedrick 2004); this system could be a powerful tool to detect KHV at low levels in water. In this study, the occurrence of KHV in river water in Japan was investigated using a virus concentration method developed in our previous study (Haramoto, Katayama & Ohgaki 2004) in concert with a real-time PCR system (Gilad *et al.* 2004).

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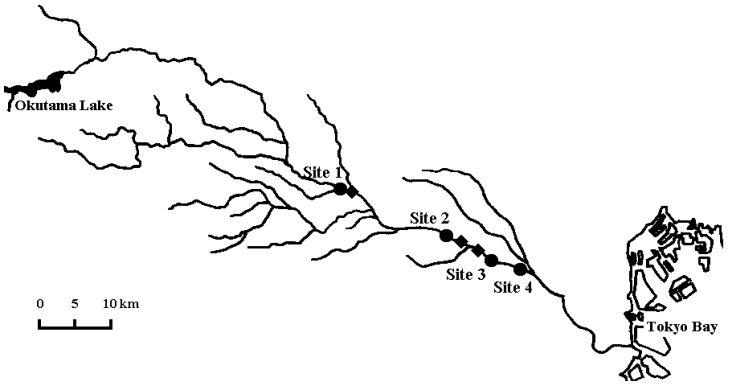


**Figure 1** Map of the Tamagawa River basin: ●, sampling sites; ◆, sites where outbreaks of koi herpesvirus occurred in May 2004.

Forty-eight river water samples (500 mL each) were collected from four sites (sites 1–4) along the Tamagawa River monthly for 1 year, from April 2003 to March 2004. The Tamagawa River has a total length of 138 km and a catchment area of 1240 $km^2$, and there are many carp in the midstream (sites 1 and 2) and downstream areas (sites 3 and 4). Figure 1 shows the sampling sites together with the sites where KHV-positive dead carp were collected. The samples were stored in plastic bottles on ice and delivered to the laboratory within 6 h after collection. The water temperature and pH of the samples were determined on site.

The water samples were concentrated using a cation ($Al^{3+}$)-coated filter method as follows (Haramoto *et al.* 2005). Briefly, 2 mL of 250 mM $AlCl_3$ were passed through an HA electronegative filter (0.45 μm pore size and 90 mm diameter; Millipore, Tokyo, Japan) attached to a glass filter holder (Advantec, Tokyo, Japan) to form a cation-coated filter, which was used to filter 500 mL of the water sample. The filter was rinsed with 200 mL of 0.5 mM $H_2SO_4$ and eluted with 5.0 mL of 1.0 mM NaOH into a tube containing 25 μL of 100 mM $H_2SO_4$ and 50 μL of 100X TE buffer.

The concentrated samples were then ultracentrifuged using a Centriprep YM-50 (Millipore) and DNA extracted using a QIAamp DNA mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocols.

The extracted DNA samples were used as templates for TaqMan PCR amplification with KHV-specific primer pairs and probe (Gilad *et al.* 2004), and amplification products were detected using an ABI PRISM 7500 sequence detection system (Applied Biosystems, Tokyo, Japan). Three wells were used for the detection of KHV-DNA for one sample. A positive result was obtained when at least one of the three wells showed DNA amplification.

Two hundred microlitres of 700 μL of the concentrated sample was used for DNA extraction, and 15 μL of 200 μL of the extracted DNA was used for the detection of KHV. Therefore, volumes tested for the presence of KHV-DNA were equivalent to 10.7 mL of the original water sample.

Table 1 shows water quality data for river water samples and the results of KHV-DNA detection in those samples. The KHV-DNA was detected in two (4.2%) of 48 samples; the samples collected from site 2 in January 2004 and from site 3 in February 2004. For each KHV-DNA-positive sample, only one of the three wells showed a positive signal. Thus, KHV seemed to be present in the river water at low levels.

In the Tamagawa River basin, the first outbreak of KHV was reported in May 2004, ultimately resulting in the death of approximately 8000 carp. This was the first large-scale mortality of carp in the Tamagawa River. As illustrated in Fig. 1, KHV-DNA was detected in dead carp collected from three sites in midstream and downstream areas of the river in May 2004. The positive results for KHV-DNA in the Tamagawa River demonstrated that KHV was present in the river for at least 4 months prior to the observed outbreaks.

The water temperature of the KHV-DNA-positive samples was 11 °C, which is outside the range of suitable water temperatures for the growth of KHV (Gilad *et al.* 2003). Therefore, it would take several months for KHV outbreaks to become apparent.

The detection of KHV-DNA by PCR may not always be indicative of the presence of infectious KHV, but monitoring for KHV-DNA in water could be a reliable tool to ensure that a water body is free from KHV.

To our knowledge, this is the first study demonstrating the occurrence of KHV-DNA in

 

**Table 1** Water quality parameters of river water samples and results of PCR for KHV-DNA

| Date of sampling | | Water quality parameter (range) | | Result of PCR for KHV-DNA | | | |
|---|---|---|---|---|---|---|---|
| Year | Month | Water temperature (°C) | pH | Site 1 | Site 2 | Site 3 | Site 4 |
| 2003 | April | 14–16 | 7.3–9.0 | – | – | – | – |
| | May | 15–17 | 7.0–9.1 | – | – | – | – |
| | June | 21–22 | 7.1–8.3 | – | – | – | – |
| | July | 18–22 | 7.2–8.1 | – | – | – | – |
| | August | 15–22 | 6.9–7.1 | – | – | – | – |
| | September | 18–25 | 7.5–9.1 | – | – | – | – |
| | October | 12–16 | 7.2–9.2 | – | – | – | – |
| | November | 9–14 | 7.6–8.2 | – | – | – | – |
| | December | 8–12 | 7.2–9.6 | – | – | – | – |
| 2004 | January | 9–11 | 7.6–9.2 | + | – | – | – |
| | February | 11–11 | 7.5–9.3 | – | + | – | – |
| | March | 6–13 | 7.1–8.7 | – | – | – | – |

+, positive for KHV-DNA; –, negative for KHV-DNA.
KHV, koi herpesvirus; PCR, polymerase chain reaction.

aquatic environments. We applied a cation-coated filter method, which was originally developed using poliovirus, to detect KHV in river water. The method was developed based on the electrostatic interactions between poliovirus, aluminium ions and an electronegative filter. KHV has a larger diameter than poliovirus (28 nm) and is covered with an envelope (Pokorova *et al.* 2005). Therefore, the question remains as to whether KHV is concentrated as efficiently as poliovirus. The recovery efficiency of KHV by this method should be evaluated in future studies.

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